Fas and FasL expression in human adipose tissue is related to obesity, insulin resistance, and type 2 diabetes

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Abstract: Context: Deletion of the death receptor Fas (CD95) in adipocytes of mice is associated with improved insulin sensitivity and reduced adipose tissue (AT) inflammation. Objective: Here we investigate the relationship of AT Fas with human obesity. Design and Methods: In paired samples of omental and sc AT from 256 lean and obese (including insulin-sensitive and insulin-resistant subgroups; n = 60) participants, we investigated whether Fas and Fas-ligand (FasL) mRNA expression is fat depot-specific, altered in obesity, and related to measures of AT inflammation and insulin sensitivity. In addition, AT Fas mRNA expression was measured in 16 obese patients after significant weight loss of 45 ± 6.3 kg in the context of a two-step bariatric surgery strategy. Results: Fas and FasL are significantly higher expressed in omental (OM) compared to sc AT. Fas expression correlates with body mass index (OM, r(2) = 0.44; sc, r(2) = 0.14), AT macrophage infiltration (OM, r(2) = 0.36; sc, r(2) = 0.16), and glucose infusion rate in euglycemic-hyperinsulinemic clamps (OM, r(2) = 0.17; sc, r(2) = 0.13) (P < .05 for all). FasL expression most strongly correlates with adipocyte size (OM, r(2) = 0.32; sc, r(2) = 0.17) and AT macrophage infiltration (OM, r(2) = 0.46; sc, r(2) = 0.02). Insulin-sensitive obese individuals had significantly lower Fas and FasL expression than insulin-resistant obese individuals. Significant weight loss 12 months after gastric sleeve resection is associated with a significantly reduced Fas expression in OM and sc fat depots. Conclusions: Independently of body weight, increased Fas expression may contribute to impaired insulin sensitivity and AT dysfunction in obesity. Moreover, significant weight loss reduces Fas expression in OM and sc fat depots.

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Fas and *FasL* expression in human adipose tissue are related to obesity, insulin resistance and type 2 diabetes

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Abstract

Context – Deletion of the death receptor Fas (CD95) in adipocytes of mice is associated with improved insulin sensitivity and reduced adipose tissue (AT) inflammation.

Objective - Here we investigate the relationship of AT Fas with human obesity.

Design and Methods – In paired samples of omental and subcutaneous (SC) AT from 256 lean and obese (including insulin sensitive (IS) and insulin resistant (IR) subgroups, n=60) participants, we investigated whether *Fas* and Fas-ligand (*FasL)* mRNA expression is fat depot-specific, altered in obesity and related to measures of AT inflammation and insulin sensitivity. In addition, AT *Fas* mRNA expression was measured in 16 obese patients after significant weight loss of 45±6.3kg in the context of a two-step bariatric surgery strategy.

Results – *Fas* and *FasL* are significantly higher expressed in omental compared to SC AT. *Fas* expression correlates with BMI (OM, r^2=0.44, SC, r^2=0.14), AT macrophage infiltration (OM, r^2=0.36, SC, r^2=0.16) and glucose infusion rate in euglycemic-hyperinsulinemic clamps (OM, r^2=0.17, SC, r^2=0.13) ($p<0.05$ for all). FasL expression most strongly correlates with adipocyte size (OM, r^2=0.32, SC, r^2=0.17) and AT macrophage infiltration (OM, r^2=0.46, SC, r^2=0.02). IS had significantly lower *Fas* and *FasL* expression than IR obese individuals. Significant weight loss 12 months after gastric sleeve resection is associated with a significantly reduced *Fas* expression in omental and SC fat depots.

Conclusions –Independently of body weight, increased *Fas* expression may contribute to impaired insulin sensitivity and AT dysfunction in obesity. Moreover, significant weight loss reduces *Fas* expression in omental and SC fat depots.

Introduction

The majority of patients with obesity has impaired adipose tissue (AT) function which is characterized by adipocyte hypertrophy, hypoxia, a variety of stresses and inflammatory processes within adipose tissue (1). Increased number of macrophages infiltrating adipose tissue (2-4), activation of stress kinases (5), higher expression of autophagy markers (6) as well as increased adipocyte apoptosis (7, 8) has been reported in human obesity. We and others have recently shown that inflammation of visceral adipose tissue may contribute to impaired whole body insulin sensitivity in patients with obesity (9, 10). Moreover, adipocyte apoptosis is increased in AT from both mice with diet-induced obesity and obese humans (7). In mice, genetic inactivation of Bid, a key pro-apoptotic molecule, markedly reduced adipocyte apoptosis prevented AT macrophage infiltration, and protected against the development of systemic insulin resistance suggesting that adipocyte apoptosis is a key initial event that contributes to macrophage infiltration into adipose tissue and insulin resistance associated with obesity in both mice and humans (7).

The death receptor Fas (CD95), a member of the TNF receptor family, plays a key role in promoting programmed cell death (apoptosis) and is activated by its ligand Fas-ligand (FasL). FasL is a type II membrane protein that belongs to the TNF family. Membrane-bound human FasL is converted to a soluble form by a matrix metalloproteinase-like enzyme (11). FasL is predominantly expressed in activated T cells, whereas Fas is expressed in various tissues (11).

Moreover, there is increasing evidence that Fas activation can stimulate inflammatory pathways in several tissues (11). Accordingly, the secretion of pro-inflammatory cytokines such as IL-1β, IL-6 and MCP-1 was increased after Fas activation (12), rendering it a potential key component of the inflammatory response. We have recently shown that adipocyte specific deletion of Fas improved insulin sensitivity and reduced hepatic steatosis as well as adipose tissue (AT) inflammation in high fat diet-fed mice (13). Interestingly, Fas activation in adipocytes not only stimulated the production of proinflammatory cytokines, but also interfered with insulin-stimulated glucose uptake and induced basal lipolysis suggesting a role of Fas in adipocyte function (14, 15). In the present study, we tested the hypothesis that *Fas* and *FasL* expression in omental and subcutaneous AT are related to obesity, AT inflammation and insulin sensitivity in paired samples of

omental and SC AT from 256 subjects with a wide range of obesity, body fat distribution, insulin sensitivity, and glucose tolerance in the context of a cross-sectional study. In addition, we asked whether significant weight loss after bariatric surgery changes AT expression of *Fas*.

Materials and Methods

Subjects

We included three different cohorts with a total number of 272 individuals in our study of *Fas* and *FasL* AT mRNA expression. We investigated *Fas* and *FasL* mRNA expression in paired omental and SC adipose tissue samples obtained from 196 extensively characterized Caucasian overweight (n=32), obese (n=108) and lean (n=56) men (n=74) and women (n=122) who underwent open abdominal surgery for elective cholecystectomy or explorative laparotomy as described previously (5, 6). With oral glucose tolerance tests, we identified individuals with type 2 diabetes (T2D) (n=67) or normal glucose tolerance (NGT) (n=129). Adipose tissue was either analyzed as a whole (immediately frozen in liquid nitrogen after explantation) (n=196) or after separation into isolated adipocytes and cells of the stromal vascular fraction (SVF) from omental and SC fat depots (lean, n=15; obese n=15; NGT, n=15; T2D, n=15) using previously described methods (16).

The second cohort consists of 60 obese individuals (BMI = 45±1.3 kg/m²), which have been matched for age, gender, BMI and body fat mass into either insulin sensitive (IS) or insulin resistant (IR) obesity based on the glucose infusion rate (GIR) in euglycemic-hyperinsulinemic clamp (IS, GIR>70µmol/kg/min; IR, GIR<60µmol/kg/min). The phenotypic characteristics of the latter cohort have been extensively described (9). Measurement of body fat content, abdominal visceral and SC fat area, parameters of glucose metabolism, insulin sensitivity as well as analyses of circulating parameters was performed as described (5, 6, 9).

A third cohort, 16 Caucasian morbidly obese patients (11 women, 5 men) without type 2 diabetes participated in a prospective weight loss study consisting of two bariatric procedures (Table 1). At baseline, patients underwent a sleeve gastrectomy and after ~12 months an elective second-step Roux-en-Y- gastric bypass surgery was performed. Collection of subcutaneous and omental fat

biopsies was performed both at the time of sleeve gastrectomy and approximately 12 months later during the Roux-en-Y bypass surgery. Patients with malignant diseases or acute or chronic inflammatory diseases were excluded from the study in all subcohorts. All subjects had a stable weight, defined as the absence of fluctuations of >2% of body weight for at least 3 months before surgery. All study protocols have been approved by the Ethics committee of the University of Leipzig. All participants gave written informed consent before taking part in the study.

Measurement of body fat content, glucose metabolism, insulin sensitivity

BMI was calculated as weight divided by squared height. Hip circumference was measured over the buttocks; waist circumference was measured at the midpoint between the lower ribs and iliac crest. Percentage body fat was measured by dual X-ray absorptiometry (DEXA). Abdominal visceral and subcutaneous fat areas were calculated using computed tomography (CT) scans at the level of L4–L5 in the cohort of paired visceral and subcutaneous adipose tissue donors. Three days before the OGTT, patients documented a high-carbohydrate diet in diet protocols. The OGTT was performed after an overnight fast with 75 g standardized glucose solution (Glucodex Solution 75 g; Merieux, Montreal, Canada). Venous blood samples were taken at 0, 60, and 120 min for measurements of plasma glucose concentrations. Insulin sensitivity was assessed using the HOMA-IR index or with the euglycemic-hyperinsulinemic clamp method as described previously (17). All baseline blood samples were collected between 8 and 10 am after an overnight fast. Plasma insulin, tumor necrosis factor α (TNF α) and interleukin-8 (IL-8) were measured with an enzyme immunometric assay for the IMMULITE automated analyzer (Diagnostic Products Corporation, Los Angeles, CA, USA). Other circulating parameters including monocyte chemoattractant protein-1 (MCP-1) and progranulin were measured as previously described (9).

Fas* and *FasL mRNA and* FAS *protein expression studies

Human *Fas* and *FasL* mRNA expression in adipose tissue, isolated adipocytes and cells of the stromal vascular fraction was measured by quantitative real-time RT-PCR in a fluorescent

146 temperature cycler using the TaqMan assay, and fluorescence was detected on an ABI PRISM 7000
147 sequence detector (Applied Biosystems, Darmstadt, Germany) as described (5, 6). Human *Fas* and
148 *FasL* mRNA expression was calculated relative to the mRNA expression of *18S rRNA*, all
149 determined by premixed assays on demand for Fas, FasL and 18s rRNA (Applied Biosystems,
150 Darmstadt, Germany). Fas protein expression was measured by Western blots using monoclonal anti-
151 human FAS (B-10) and normalized to glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) protein
152 (both from Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected by incubating with
153 HRP conjugated secondary antibodies at a 1:3000 dilution at RT for 2 h and chemiluminescence kit
154 (Amersham, Pharmacia, Freiburg, Germany). Integrated optical densities of the immunoreactive
155 protein bands were measured with Gel Analyzer software (Media Cyberneties, Silver Spring, MD).
156 Frequency of apoptotic cells was determined in histology sections of AT counting active caspase-3
157 positive cells as described (8).
158
159 ***Statistical analyses***
160 Data are shown as means ± SD unless stated otherwise. The following statistical tests were
161 used: paired Student's t-test, chi square test, and Pearson's simple correlation. We examined
162 independent associations between weight-loss associated changes in omental/SC *Fas* expression and
163 BMI, HbA1c, fasting plasma glucose and insulin, HOMA-IR, and circulating free fatty acids using a
164 stepwise multivariate linear regression model stratified by gender. Statistical analysis was performed
165 using SPSS version 12.0 (Chicago, IL). *P* values < 0, 05 were considered to be statistically
166 significant.
167
168 **Results**
169 ***Fas* mRNA expression correlates with parameters of obesity, adipose tissue function and**
170 **insulin sensitivity**
171 Independent of gender, *Fas* mRNA expression in whole adipose tissue was significantly
172 higher in omental compared to SC fat depot (Figure 1A) with a significant correlation between these
173 two depots ($r=0.22$, $p<0.05$). Both visceral and SC *Fas* expression was significantly higher in

patients with obesity (Figure 1B) and T2D (Figure 1C) compared to lean or NGT controls. We found significant positive correlations between omental *Fas* expression and parameters of obesity including BMI ($r=0.66$, $p<0.001$), body fat ($r=0.52$, $p<0.01$), visceral fat area ($r=0.66$, $p<0.001$), and waist circumference ($r=0.51$, $p<0.01$) (Table 1). In addition, omental *Fas* expression significantly correlates with parameters of AT function such as adipocyte size, macrophage infiltration into visceral AT, adiponectin serum concentration (Table 2), as well as with the number of apoptotic adipocytes ($r=0.64$, $p<0.001$). Moreover, omental *Fas* mRNA correlates with HbA1c, circulating IL-6, IL-8, MCP-1 and progranulin, but not TNFα (Table 2). We found significant positive correlations in obese subjects between omental *Fas* expression and circulating triglyceride (Table 2) and FFA levels ($r=0.35$, $p<0.01$). Noteworthy, only the associations between omental *Fas* expression, adipocyte hypertrophy, AT macrophage infiltration and insulin sensitivity (GIR) remained significant ($p<0.05$) after adjusting for BMI.

Subcutaneous *Fas* expression positively correlates with BMI, body fat, SC fat area, waist circumference, macrophage infiltration into SC AT, circulating IL-6, IL-8, MCP-1, progranulin, (Table 2), and importantly the number of apoptotic adipocytes ($r=0.62$, $p<0.001$). Both omental and SC *Fas* expression significantly correlate with parameters of insulin sensitivity, fasting plasma insulin, circulating adiponectin and the GIR in euglycemic-hyperinsulinemic clamps (Table 2). However, the extent of the associations between *Fas* expression and these parameters differs between the two fat depots. For example, the correlation between *Fas* (and also *FasL*) expression and circulating adiponectin is stronger for the omental compared to the SC fat depot (Tables 2 and 3).

To further elucidate the relationship between impaired insulin sensitivity and AT *Fas* expression independently of BMI or fat mass, we compared *Fas* expression in individuals with either IS or IR obesity after strictly matching for age, gender and BMI (9). Individuals with IS obesity had significantly lower *Fas* expression both in omental and SC AT than IR obese individuals (Figure 1D). Lower *Fas* expression in IS obesity is associated with significantly lower serum concentrations of IL-6, IL-8, progranulin and MCP-1 (9), which could either be the consequence or contribute to lower *Fas* expression in the IS compared to IR healthy obese subgroup.

We further sought to dissect the contribution of adipocytes and cells of the stromal vascular fraction (SVF) on *Fas* expression in adipose tissue. Expression analyses of representative subgroups (n=15 per group) revealed that both isolated adipocytes and cells of the SVF contribute to differences in adipose tissue *Fas* expression between omental and subcutaneous fat depots, lean and obese as well as individuals with NGT or T2D (Figure 1E, F).

In parallel to *Fas* mRNA expression data, we found significantly higher Fas protein levels in omental compared to SC AT as well as significantly higher omental Fas protein levels in patients with obesity and type 2 diabetes compared to lean controls (Figure 1G).

Reduced *Fas* mRNA expression after significant weight loss

We aimed to analyze changes in AT *Fas* mRNA expression in the context of a two-step bariatric surgery strategy with an initial gastric sleeve followed by a gastric bypass surgery after ~12 months. In response to bariatric surgery, we found significantly decreased BMI, improved insulin sensitivity as measured by HOMA-IR and fasting plasma insulin and reduced fasting plasma glucose, HbA1c and free fatty acids (Table 1). We found ~40% lower omental *Fas* mRNA expression 12 months after significant weight loss induced by the initial bariatric surgery (Figure 2A). In parallel, SC *Fas* mRNA expression decreased significantly by 33% (Figure 2B). By stepwise multivariate regression models stratified for gender including BMI, HbA1c, fasting plasma glucose and insulin, HOMA-IR, and circulating free fatty acids , we identified changes in BMI as strongest predictor of decreased omental and SC *Fas* expression ($p<0.01$).

***FasL* mRNA expression is associated with adipocyte hypertrophy and macrophage infiltration into AT**

In addition to *Fas* mRNA expression, we sought to determine *FasL* expression in omental and SC AT. Interestingly, *FasL* mRNA was only detectable in ~60% of the AT samples (omental: n=118/196; SC: n=116/196) of the cross sectional study and in less then 50% of the IS/IR obesity subgroup (IS: omental: n=14/30; SC: n=14/30; IR: omental: n=16/30; SC: n= 14/30). Age, gender

distribution, diagnosis of type 2 diabetes, BMI, parameters of fat distribution, insulin sensitivity, adipose tissue biology, glucose or lipid metabolism were not significantly different between patients with or without detectable omental and/or SC *FasL* expression (data not shown).

In study participants with detectable *FasL* expression, we find significantly higher *FasL* expression in omental compared to SC AT (Figure 3A). This difference was independent of gender, BMI group and the diagnosis of type 2 diabetes. *FasL* expression correlates between the two fat depots (r^2=0.29, $p<0.001$). In parallel to *Fas* expression, both visceral and SC *FasL* expression was significantly higher in patients with obesity (Figure 3B) and T2D (Figure 3C) compared to lean or NGT controls. Omental *FasL* expression significantly correlates with BMI, % body fat, waist circumference, visceral fat area, fasting plasma insulin, GIR, circulating triglycerides, adiponectin, IL-6, IL-8, MCP-1, progranulin, adipocyte size and number of macrophages in visceral AT (Table 3). In contrast, SC *FasL* expression is only significantly associated with BMI, waist circumference, circulating adiponectin, adipocyte size and AT infiltrating macrophages (Table 3).

The relationships between omental *FasL* expression, adipocyte hypertrophy, AT macrophage infiltration and insulin sensitivity (GIR) remained significant ($p<0.05$) after adjusting for BMI, whereas only the relationship between SC *FasL* mRNA and adipocyte size was independent of BMI ($p<0.05$). Patients with IR obesity have significantly higher *FasL* expression in omental, but not in SC AT than IS obese individuals (Figure 3D). To investigate the contribution of different AT cell types on *FasL* expression, we performed additional analyses on *FasL* expression in isolated adipocytes and SVF cells separately from representative subgroups (n=15 per group) of lean versus obese and NGT versus T2D in omental and SC fat. Importantly, we found in both, adipocytes and cells of the SVF, parallel differences as observed for adipose tissue as a whole (Figure 3 E, F).

Discussion

In this study, we find significant relationships between AT *Fas* expression and parameters of obesity (BMI, body fat mass), fat distribution (waist circumference, visceral and SC fat area), adipose tissue function (macrophage infiltration, adipocyte size, adiponectin serum concentration), adipocyte apoptosis and insulin sensitivity (fasting plasma insulin, clamp GIR) in humans. In adipose

tissue as a whole, but also in isolated adipocytes and cells of the SVF, *Fas* expression was significantly higher in omental compared to SC, in obese versus lean individuals and T2D compared to NGT patients. These data suggest that both changes in adipocyte and SVF cell *Fas* expression contribute to the observed group differences and correlations associated with adipose tissue *Fas* expression. Further studies are required to quantify the exact contribution of individual cell types within adipose tissue on altered *Fas* expression observed in obesity and metabolic diseases. We thereby extend previous findings in mice with an adipocyte-specific Fas- knockout (AFasKO), which are protected against HFD-induced insulin resistance (13). We demonstrate both with statistical methods and the human model system of insulin sensitive obesity (9) that associations between omental *Fas* expression, macrophage infiltration and insulin sensitivity are independent of BMI. Our findings thus suggest that Fas-mediated pathways in adipocytes may link adipose tissue dysfunction to impaired insulin sensitivity at the whole body level beyond simple associations between increased fat mass and insulin resistance.

Increased AT *Fas* expression may be an important factor in the development of AT dysfunction. Fas is a proapoptotic factor, and adipocyte hypertrophy in obesity was proposed to promote adipocyte cell death, possibly with some apoptotic characteristics (18). When AT hypertrophy develops in obesity, Fas may be induced and thereby contribute to enhanced AT apoptosis, which may attract immune cells into adipose tissue. The latter may be the consequence of increased Fas-induced chemokine production as was recently shown for other cell types (19). Indeed, we found increased Fas protein levels in hypertrophic compared to small adipocytes (13). Moreover, expression of chemotactic factors such as MCP-1 was reduced in adipose tissue of AFasKO mice, as was CD11b expression, suggesting reduced immune cell attraction (13). In accordance with that, we found significant correlations between serum concentrations of the chemoattractant cytokines IL-6, IL-8, MCP-1 and progranulin and both *Fas* and *FasL* expression. However, these correlations did not remain significant after adjusting for age, gender and BMI. Thus, increased *Fas* expression in hypertrophic adipocytes could be an early event in the development of AT dysfunction in humans and contribute to insulin resistant obesity.

We found significant negative correlations between *Fas* and *FasL* expression and adiponectin serum concentrations, which are consistently stronger in the omental compared to SC fat depot. We included circulating adiponectin as a covariate in our study, because it has been shown that adiponectin inhibits up-regulation of Fas induced during the development of hepatic steatosis (20). Maybe due to structural similarities between the two molecules, adiponectin antagonizes many effects of TNFα and low adiponectin concentrations may contribute to increased *Fas* and *FasL* expression in obesity. Therefore, dysproportional association between adiponectin and *Fas* or *FasL* expression in the different depots may be explained by previously demonstrated lower adiponectin expression in visceral compared to SC fat depots (21).

Increased apoptosis of adipocytes has been associated with obesity in both rodents and humans and may represent one of the early mechanisms causing macrophage infiltration with the subsequent development of systemic subclinical inflammation and impaired insulin sensitivity (7). In a cross-sectional study of HIV type 1-infected patients after nucleoside analogue reverse-transcriptase inhibitor therapy, AT dysfunction and lipoatrophy was associated with increased apoptosis (independently of obesity) and an up to 7-fold higher number of AT macrophages compared to non-lipoatrophic HIV-1-infected patients (22). In accordance with our data, Tinahones et al. (23) recently found that a proapoptotic state characterized by increased expression of caspase 3/7 and BCL2 in AT of obese humans correlates with increased immune cell infiltration into AT and impaired insulin signaling. Of course, from these cross-sectional data, it is difficult to establish a potential sequence from increased Fas expression to increased apoptosis, attraction of immune cells into AT with subsequent AT dysfunction. However, these (together with our) data are at least suggestive that activation of adipocyte apoptosis in obesity contributes to the development of insulin resistance. However, the factors protecting a subgroup of healthy insulin sensitive, but extremely obese patients (9) against such proapoptotic state need to be explored. One explanation could be that the significantly smaller adipocytes (both mean and maximal size) in insulin sensitive compared to insulin resistant obese (9) are less likely to enter apoptosis. This hypothesis is supported by data from obese mice and humans demonstrating that the frequency of apoptosis is positively correlated with increased adipocyte size (24). Moreover, >90% of macrophages in AT co-localize with dead or

apoptotic adipocytes further suggesting that apoptosis may initiate the attraction of immune cells into AT (24). Adipose tissue has a remarkable capacity to expand in a "benign" non-neoplastic manner. Increased adipocyte hypertrophy, AT dysfunction and obesity related metabolic diseases maybe the result of an impaired capacity to store even more fat (22). In many obese patients SC AT expandability is impaired, this fat depot reaches its maximal storage capacity and hypertrophied adipocytes may enter a proapoptotic state (25). Reduced *Fas* expression after significant weight loss upon our bariatric surgery intervention suggests that the proapoptotic state may be a reversible condition. However, since weight loss was only examined in patients following bariatric surgery, we can not exclude potential effects on adipose tissue gene expression by the surgery independently of weight loss alone. Therefore, it would be interesting to examine changes in *Fas* and *FasL* gene expression of individuals that lost weight without surgical intervention. Further moderate weight loss interventions are required to define whether the amount of weight loss and/or predominantly visceral AT reduction may reverse increased expression of proapoptotic molecules in AT.

Fas activation may also exert direct metabolic effects unrelated to cell death, similar to the endocrine effects of TNFα (26). Fas activation interfered with insulin-stimulated glucose uptake via down-regulation of Akt (14), increased secretion of pro-inflammatory cytokines (19) and enhanced lipolysis independent of apoptosis in 3T3-L1 adipocytes at least in vitro (13, 15). Supporting the latter notion, we recently reported that Fas activation increased phosphorylation of ERK1/2, and FasL-induced lipolysis was blunted in the presence of the ERK-inhibitor U0126 or in ERK1/2-depleted adipocytes (15). Similarly, we show here that in obese humans subjects, omental *Fas* mRNA expression positively correlates with adipocyte volume and circulating FFA levels, suggesting that increased *Fas* expression in (hypertrophic) adipocytes may contribute to increased basal lipolysis (and hence FFA levels) in humans.

In individuals with insulin sensitive obesity, factors underlying the protection against increased *Fas* expression despite extreme obesity need to be explored. Interestingly, correlations between *FasL* expression and parameters of obesity, fat distribution, AT function and glucose metabolism closely reflect the associations observed for AT *Fas* Expression. Similar to *Fas* expression, both adipocytes and cells of SVF contribute to adipose tissue *FasL* expression. However,

only in 50-60% of the AT donors in our study, we detected *FasL* mRNA expression suggesting that increased Fas expression represents an earlier detectable factor than increased *FasL* in the association with phenotypes like obesity, fat distribution, adipose tissue function and insulin sensitivity.

In conclusion, we show here that independently of body weight, increased *Fas* expression may contribute to impaired insulin sensitivity and AT dysfunction in obesity. Our data support an important role of Fas in the initiation of adipose tissue inflammation and dysfunction and suggest that reduced AT *Fas* expression may contribute to improved insulin sensitivity and subclinical inflammation despite obesity.

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Author contributions

M.B.: research data and wrote manuscript, N.K.: research data, S.W.: research data and edit manuscript. E.J.S.: edit manuscript, contributed to discussion, M.R.S.: research data, A.D.: research data, M.F. edit manuscript, M.S. edit manuscript, contributed to discussion, D. K.: research data and wrote manuscript

References

1. **Blüher M** 2009 Adipose tissue dysfunction in obesity. Exp Clin Endocrinol Diabetes 117:241-250
2. **Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr** 2003 Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112: 1796-1808
3. **Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, Bouillot JL, Bouloumié A, Barbatelli G, Cinti S, Svensson PA, Barsh GS, Zucker JD, Basdevant A, Langin D, Clément K** 2005 Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. Diabetes 54: 2277–2286
4. **Harman-Boehm I, Blüher M, Redel H, Sion-Vardy N, Ovadia S, Avinoach E, Shai I, Klöting N, Stumvoll M, Bashan N, Rudich A** 2007 Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity. J Clin Endocrinol Metab 92: 2240-2247
5. **Blüher M, Bashan N, Shai I, Harman-Boehm I, Tarnovscki T, Avinaoch E, Stumvoll M, Dietrich A, Klöting N, Rudich A** 2009 Activated Ask1-MKK4-p38MAPK/JNK stress signaling pathway in human omental fat tissue may link macrophage infiltration to whole-body Insulin sensitivity. J Clin Endocrinol Metab 94: 2507-2515
6. **Kovsan J, Blüher M, Tarnovscki T, Klöting N, Kirshtein B, Madar L, Shai I, Golan R, Harman-Boehm I, Schön MR, Greenberg AS, Elazar Z, Bashan N, Rudich A** 2011 Altered autophagy in human adipose tissues in obesity. J Clin Endocrinol Metab 96: E268-77
7. **Alkhouri N, Gornicka A, Berk MP, Thapaliya S, Dixon LJ, Kashyap S, Schauer PR, Feldstein AE** 2010 Adipocyte apoptosis, a link between obesity, insulin resistance, and hepatic steatosis. J Biol Chem 285: 3428-3438
8. **Keuper M, Blüher M, Schön MR, Möller P, Dzyakanchuk A, Amrein K, Debatin KM, Wabitsch M, Fischer-Posovszky P** 2011 An inflammatory micro-environment promotes human adipocyte apoptosis. Mol Cell Endocrinol 339:105-113

9. **Klöting N, Fasshauer M, Dietrich A, Kovacs P, Schön MR, Kern M, Stumvoll M, Blüher M** 2010 Insulin-sensitive obesity. Am J Physiol Endocrinol Metab 299: E506-15
10. **Hardy OT, Perugini RA, Nicoloro SM, Gallagher-Dorval K, Puri V, Straubhaar J, Czech MP** 2011 Body mass index-independent inflammation in omental adipose tissue associated with insulin resistance in morbid obesity. Surg Obes Relat Dis 7:60-67
11. **Peter ME, Budd RC, Desbarats J, Hedrick SM, Hueber AO, Newell MK, Owen LB, Pope RM, Tschopp J, Wajant H, Wallach D, Wiltrout RH, Zörnig M, Lynch DH** 2007 The CD95 receptor: apoptosis revisited. Cell 129: 447–450
12. **Wajant H, Pfizenmaier K, Scheurich P** 2003 Non-apoptotic Fas signaling. Cytokine Growth Factor Rev 14: 53-66
13. **Wueest S, Rapold RA, Schumann DM, Rytka JM, Schildknecht A, Nov O, Chervonsky AV, Rudich A, Schoenle EJ, Donath MY, Konrad D** 2010 Deletion of Fas in adipocytes relieves adipose tissue inflammation and hepatic manifestations of obesity in mice. J Clin Invest 120: 191-202
14. **Wueest S, Rapold RA, Schoenle EJ, Konrad D** 2010 Fas activation in adipocytes impairs insulin-stimulated glucose uptake by reducing Akt. FEBS Lett 584: 4187-4192
15. **Rapold RA, Wueest S, Knoepfel A, Schoenle EJ, Konrad D** 2013 Fas activates lipolysis in a Ca2+-CaMKII-dependent manner in 3T3-L1 adipocytes. J Lipid Res 54:63-70
16. **Gesta S, Blüher M, Yamamoto Y, Norris AW, Berndt J, Kralisch S, Boucher J, Lewis C, Kahn CR** 2006 Evidence for a role of developmental genes in the origin of obesity and body fat distribution. Proc Natl Acad Sci U S A 103: 6676-6681
17. **Blüher M, Unger R, Rassoul F, Richter V, Paschke R** 2002 Relation between glycaemic control, hyperinsulinaemia and plasma concentrations of soluble adhesion molecules in patients with impaired glucose tolerance or type II diabetes. Diabetologia 45: 210 –216
18. **Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS** 2005 Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res 46:2347–2355

19. **Cullen SP, Henry CM, Kearney CJ, Logue SE, Feoktistova M, Tynan GA, Lavelle EC, Leverkus M, Martin SJ** 2013 Fas/CD95-Induced Chemokines Can Serve as "Find-Me" Signals for Apoptotic Cells. Mol Cell 49: 1034-1048

20. **Wedemeyer I, Bechmann LP, Odenthal M, Jochum C, Marquitan G, Drebber U, Gerken G, Gieseler RK, Dienes HP, Canbay A** 2009 Adiponectin inhibits steatotic CD95/Fas up-regulation by hepatocytes: therapeutic implications for hepatitis C. J Hepatol 50: 140-149

21. **Lihn AS, Bruun JM, He G, Pedersen SB, Jensen PF, Richelsen B** 2004 Lower expression of adiponectin mRNA in visceral adipose tissue in lean and obese subjects. Mol Cell Endocrinol 219: 9-15

22. **Sievers M, Walker UA, Sevastianova K, Setzer B, Wågsäter D, Eriksson P, Yki-Järvinen H, Sutinen J** 2009 Gene expression and immunohistochemistry in adipose tissue of HIV type 1-infected patients with nucleoside analogue reverse-transcriptase inhibitor-associated lipoatrophy. J Infect Dis 200:252-262

23. **Tinahones FJ, Coín Aragüez L, Murri M, Oliva Olivera W, Mayas Torres MD, Barbarroja N, Gomez Huelgas R, Malagón MM, El Bekay R** 2013 Caspase Induction and BCL2 Inhibition in Human Adipose Tissue: A potential relationship with insulin signaling alteration. Diabetes Care 36:513-521

24. **Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS** 2005 Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res 46: 2347-2355

25. **Tan CY, Vidal-Puig A** 2008 Adipose tissue expandability: the metabolic problems of obesity may arise from the inability to become more obese. Biochem Soc Trans 36: 935-40

26. **Hotamisligil GS** 2005 Role of endoplasmic reticulum stress and c-Jun NH2-terminal kinase pathways in inflammation and origin of obesity and diabetes. Diabetes 54: S73–S78

Figure legends

Figure 1. *Fas* mRNA expression and protein levels in human omental (OM) and subcutaneous (SC) adipose tissue.

(A) *Fas* mRNA expression in the two different fat depots in the entire study cohort (n=256). (**B**) Omental (OM) and subcutaneous (SC) *Fas* mRNA expression in lean (BMI< 25kg/m², n=56) and obese (BMI>30kg/m², n=93) individuals. (**C**) Omental (OM) and subcutaneous (SC) *Fas* mRNA expression in normal glucose tolerant (NGT) individuals (n=129) and patients with type 2 diabetes (T2D) (n=67). (**D**) Omental (OM) and subcutaneous (SC) *Fas* mRNA expression in individuals with insulin sensitive (IS) or insulin resistant (IR) obesity. IS and IR groups were matched for age, gender, BMI and body fat mass. (**E**) *Fas* mRNA expression in isolated adipocytes from omental (OM) and subcutaneous (SC) adipose tissue of lean (BMI< 25kg/m², n=15) and obese (BMI>30kg/m², n=15), NGT (n=15) and T2D (n=15) individuals. (**F**) *Fas* mRNA expression in cells of the stromal vascular fraction (SVF) from omental (OM) and subcutaneous (SC) adipose tissue of lean (BMI< 25kg/m², n=15) and obese (BMI>30kg/m², n=15), NGT (n=15) and T2D (n=15) individuals.
(**G**) Representative Western blots for SC and omental Fas protein level in lean, obese and T2D individuals (n=2 per group) and Fas protein levels each normalized for GAPDH protein content in SC and omental adipose tissue from lean (n=11), obese (n=9), and patients with T2D (n=7). Integrated optical densities of the immunoreactive protein bands were measured with Gel Analyzer software (Media Cyberneties, Silver Spring, MD). *p<0.05, ***p<0.001 for the difference between OM and SC fat depots. # p<0.05 for the differences between the groups. Data are means +/- SEM. *p<0.05 compared to the lean group.

Figure 2. Changes of *Fas* mRNA expression in response to a significant weight loss 12 months after sleeve gastrectomy surgery.

(A) Omental adipose tissue *Fas* expression; **(B)** Subcutaneous adipose tissue *Fas* expression. Data are means +/- SEM. *p<0.05, **p<0.01 compared to baseline.

Figure 3. *FasL* mRNA expression in human omental and subcutaneous (SC) adipose tissue. (A) *FasL* mRNA expression in the two different fat depots in those study participants with detectable *FasL* expression (omental: n=118; SC: n=116). (**B**) Omental and subcutaneous (SC) *FasL* mRNA expression in lean (BMI< 25kg/m², n=24) and obese (BMI>30kg/m², n=62) individuals. (**C**) Omental and subcutaneous (SC) *FasL* mRNA expression in normal glucose tolerant (NGT) individuals (n=67) and patients with type 2 diabetes (T2D) (n=38). (**D**) Omental and subcutaneous (SC) *FasL* mRNA expression in individuals with insulin sensitive (IS, n=14 for both depots) or insulin resistant (IR; omental: n=16; SC: n=14) obesity. IS and IR groups were matched for age, gender, BMI and body fat mass. (**E**) *FasL* mRNA expression in isolated adipocytes from omental (OM) and subcutaneous (SC) adipose tissue of lean (BMI< 25kg/m², n=15) and obese (BMI>30kg/m², n=15), NGT (n=15) and T2D (n=15) individuals. (**F**) *FasL* mRNA expression in cells of the stromal vascular fraction (SVF) from omental (OM) and subcutaneous (SC) adipose tissue of lean (BMI< 25kg/m², n=15) and obese (BMI>30kg/m², n=15), NGT (n=15) and T2D (n=15) individuals.

*p<0.05, ***p<0.001 for the difference between OM and SC fat depots. # p<0.05; ### p<0.001 for the differences between the groups. Data are means +/- SEM.

Table 1. Effects of a sleeve gastrectomy on anthropometric and metabolic parameters at baseline and after 12±1.3 months, the time point of the second step bariatric surgery (gastric bypass). Data are expressed as mean ± SEM. *p<0.05, ** p<0.01, ***p<0.001 compared to baseline. FPG, fasting plasma glucose, FPI, fasting plasma insulin, FFA, free fatty acids

	Two-step bariatric surgery intervention	
	Baseline **Sleeve gastrectomy**	**12 ± 1.3 months** **Gastric bypass**
Male/female	5/11	
Age (years)	46 ± 5.8	
BMI (kg/m²)	58.9 ± 9.6	44.1 ± 6.2***
HbA1c (%)	5.7 ± 0.2	5.2 ± 0.3*
FPG (mmol/l)	5.8 ± 0.4	5.3 ± 0.5*
FPI (mmol/l)	116 ± 42	47 ± 17**
HOMA-IR	4.3 ± 0.7	1.6 ± 0.3**
FFA (mmol/l)	0.52 ± 0.1	0.28 ± 0.07*

Table 2. Univariate correlations (Spearman) between *Fas* mRNA expression in omental and subcutaneous (SC) adipose tissue and measures of obesity, insulin sensitivity, and parameters of inflammation. GIR, glucose infusion rate during the steady state of an euglycemic-hyperinsulinemic clamp; r, Spearman´s correlation coefficient. ns, not significant

	Omental *Fas* mRNA		**SC *Fas* mRNA**	
	(n=196)		**(n=196)**	
	r	**p-value**	**r**	**p-value**
Age	0.03	ns	0.02	ns
BMI	0.56	<0.001	0.25	<0.05
% body fat	0.43	<0.001	0.22	<0.05
Waist circumference	0.45	<0.001	0.37	<0.01
Visceral fat area	0.51	<0.001	0.38	<0.01
SC fat area	0.24	<0.05	0.35	<0.01
Fasting plasma glucose	-0.1	ns	-0.09	ns
HbA1c	0.19	0.04	0.12	ns
Fasting plasma insulin	0.27	<0.05	0.29	<0.05
GIR	-0.34	<0.01	-0.26	<0.05
Triglycerides	0.25	<0.05	0.24	<0.05
Adiponectin	-0.52	<0.001	-0.3	<0.01
Mean adipocyte size	0.6	<0.001	0.34	<0.01
IL-6	0.37	<0.01	0.37	<0.001
IL-8	0.22	<0.05	0.18	<0.05
MCP-1	0.31	<0.01	0.33	<0.01
Progranulin	0.39	<0.01	0.32	<0.01
TNFα	0.1	ns	0.12	ns
% macophage in adipose tissue	0.43	<0.001	0.33	<0.01

Table 3. Univariate correlations (Spearman) between *FasL* mRNA expression in omental and subcutaneous (SC) adipose tissue and measures of obesity, insulin sensitivity, and parameters of inflammation. GIR, glucose infusion rate during the steady state of an euglycemic-hyperinsulinemic clamp; r, Spearman´s correlation coefficient. ns, not significant

	Omental *FasL* mRNA (n=118)		**SC *FasL* mRNA (n=116)**	
	r	**p-value**	**r**	**p-value**
Age	0.1	ns	0.1	ns
BMI	0.38	<0.01	0.25	<0.05
% body fat	0.24	<0.05	0.12	ns
Waist circumference	0.43	<0.001	0.3	<0.05
Visceral fat area	0.63	<0.001	0.35	<0.01
SC fat area	0.01	ns	0.17	ns
Fasting plasma glucose	-0.1	ns	-0.17	ns
HbA1c	0.2	ns	-0.03	ns
Fasting plasma insulin	0.22	<0.05	-0.05	ns
GIR	-0.47	<0.001	-0.11	ns
Triglycerides	0.29	<0.01	0.11	ns
Adiponectin	-0.46	<0.001	-0.2	<0.05
Mean adipocyte size	0.57	<0.001	0.41	<0.001
IL-6	0.4	<0.001	0.18	ns
IL-8	0.31	<0.05	0.12	ns
MCP-1	0.25	<0.05	0.09	ns
Progranulin	0.21	<0.05	0.08	ns
TNFα	0.08	ns	0.06	ns
% macophage in adipose tissue	0.68	<0.001	0.2	<0.05

Figure 1

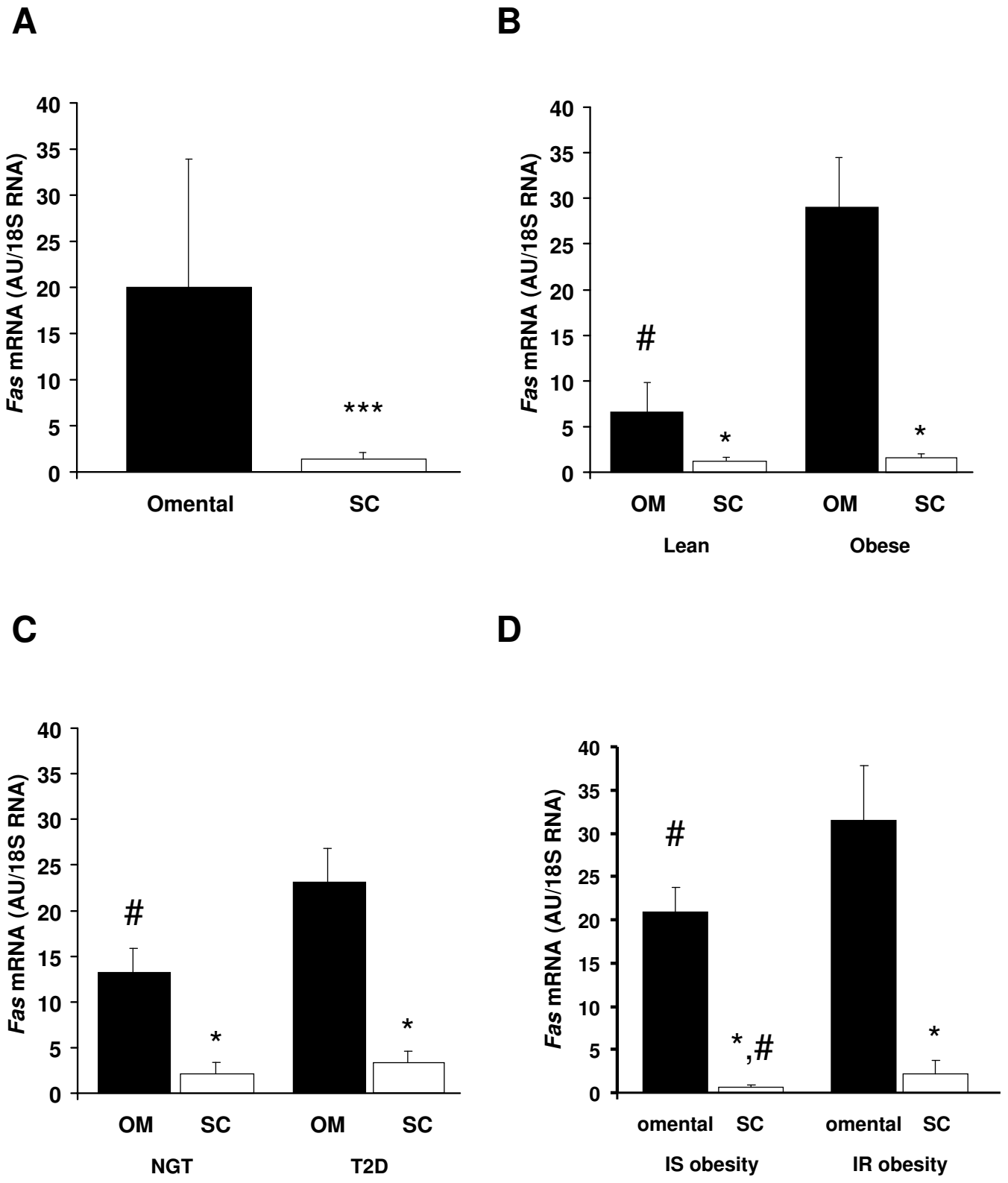
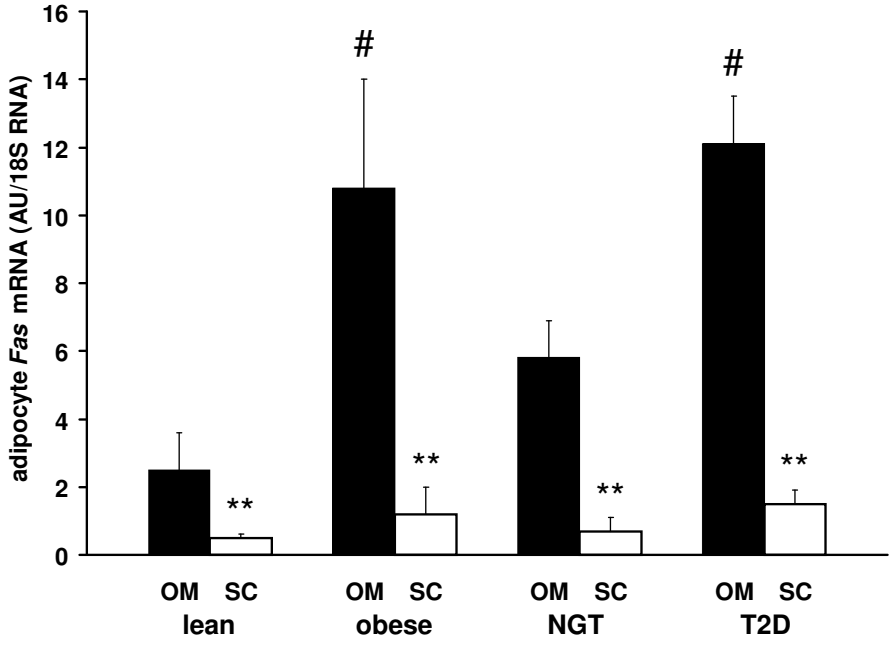

A
B
C
D
Fas mRNA (AU/18S RNA)
40
35
30
25
20
15
10
5
0
***
Omental
SC
#
*
OM
SC
Lean
Obese
NGT
T2D
*,#
omental
IS obesity
IR obesity

Figure 1

E


adipocyte Fas mRNA (AU/18S RNA)
16
14
12
10
8
6
4
2
0
#
#
**
**
**
**
OM SC
lean
OM SC
obese
OM SC
NGT
OM SC
T2D

F

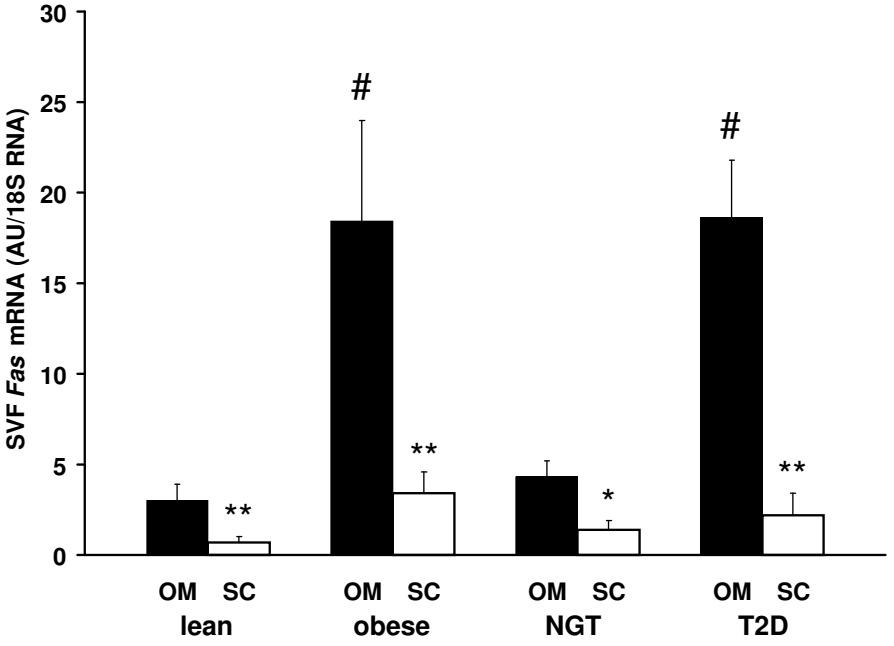

SVF Fas mRNA (AU/18S RNA)
30
25
20
15
10
5
0
#
#
**
**
*
**
OM SC
lean
OM SC
obese
OM SC
NGT
OM SC
T2D

Figure 1

G

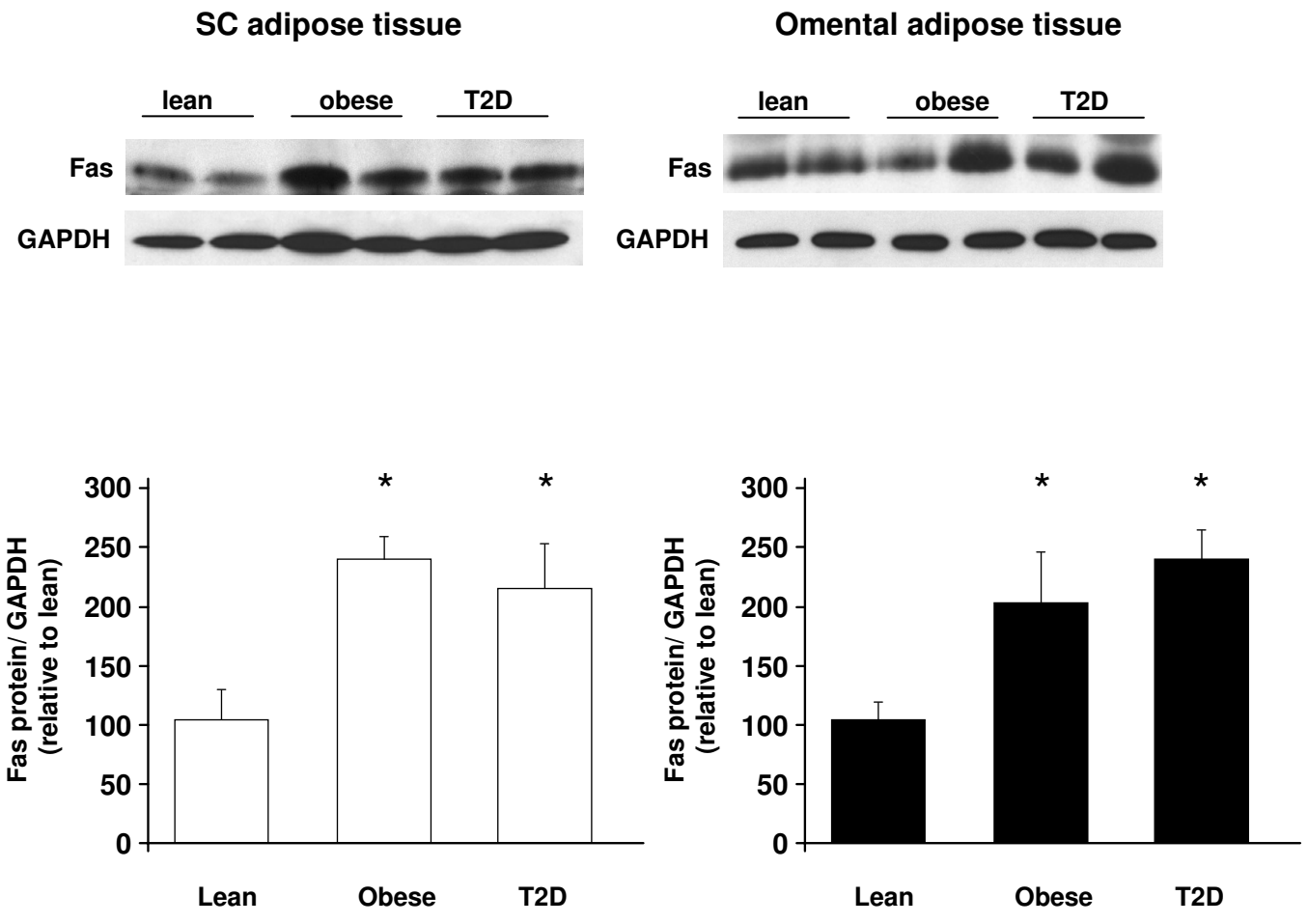

SC adipose tissue
Omental adipose tissue
lean
obese
T2D
Fas
GAPDH
Fas protein/ GAPDH (relative to lean)
300
250
200
150
100
50
0
*
Lean
Obese
T2D

Figure 2

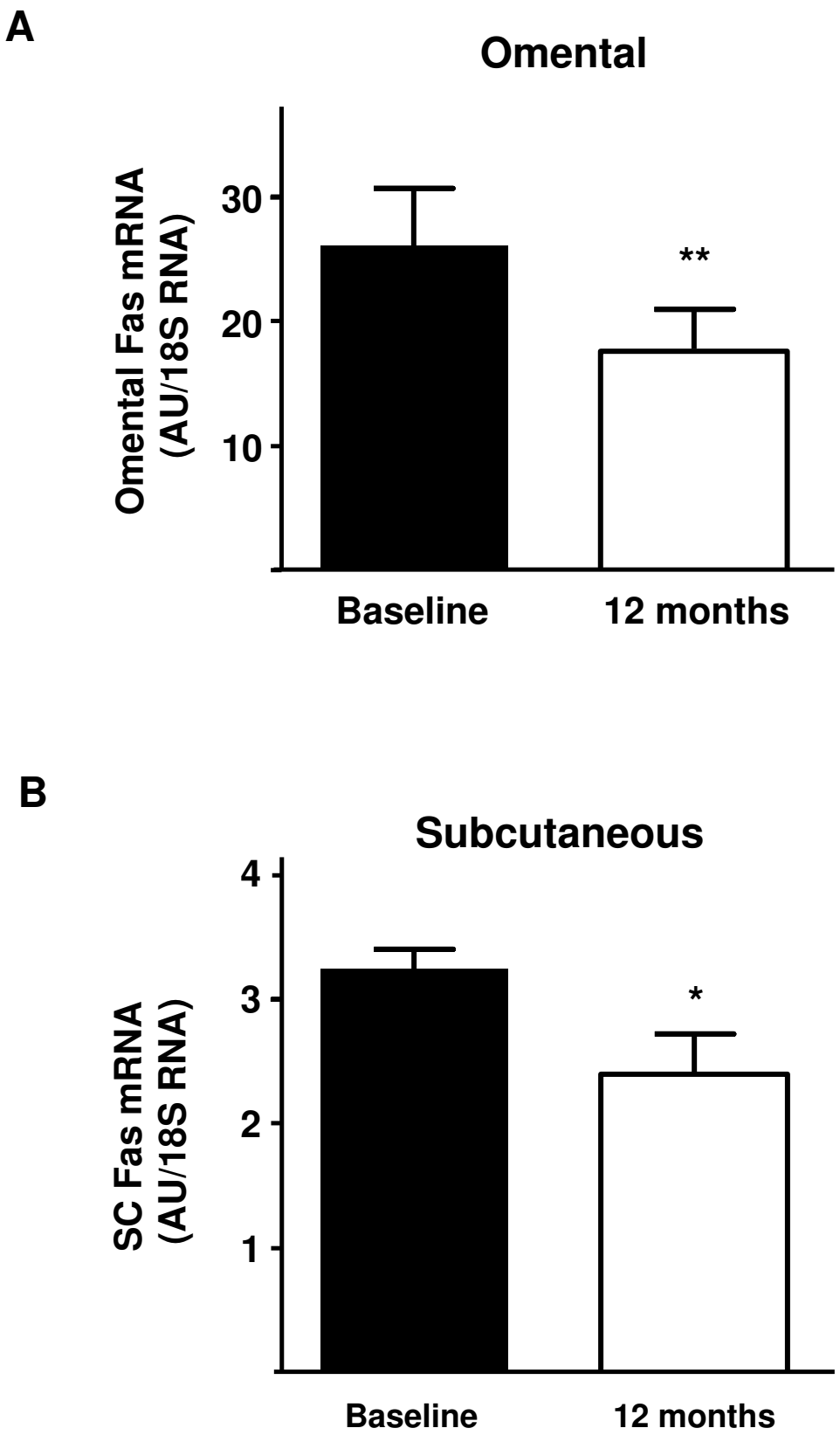
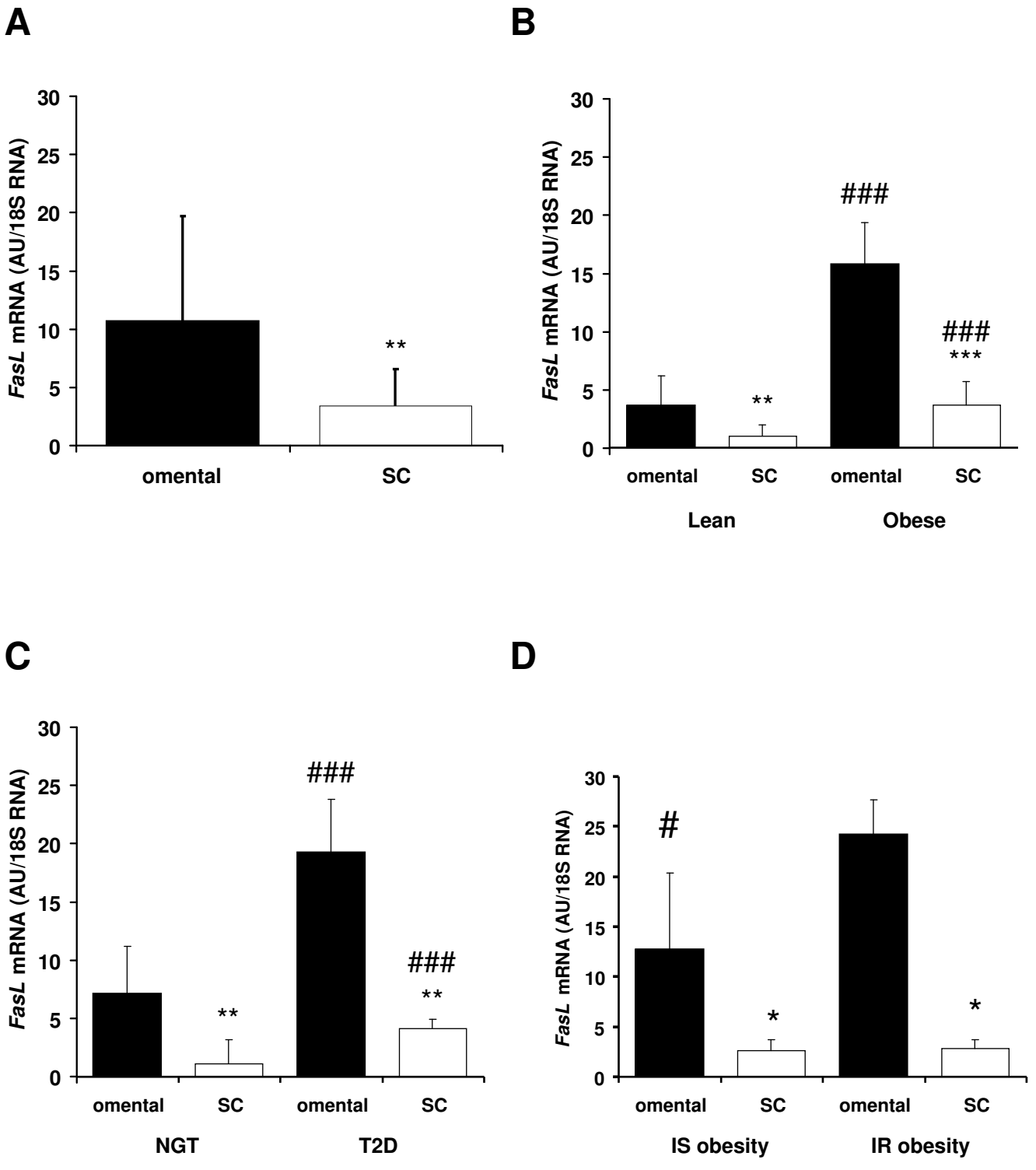

A
Omental
Omental Fas mRNA (AU/18S RNA)
30
20
10
**
Baseline
12 months
B
Subcutaneous
SC Fas mRNA (AU/18S RNA)
4
3
2
1
*
Baseline
12 months

Figure 3


A
B
C
D
FasL mRNA (AU/18S RNA)
30
25
20
15
10
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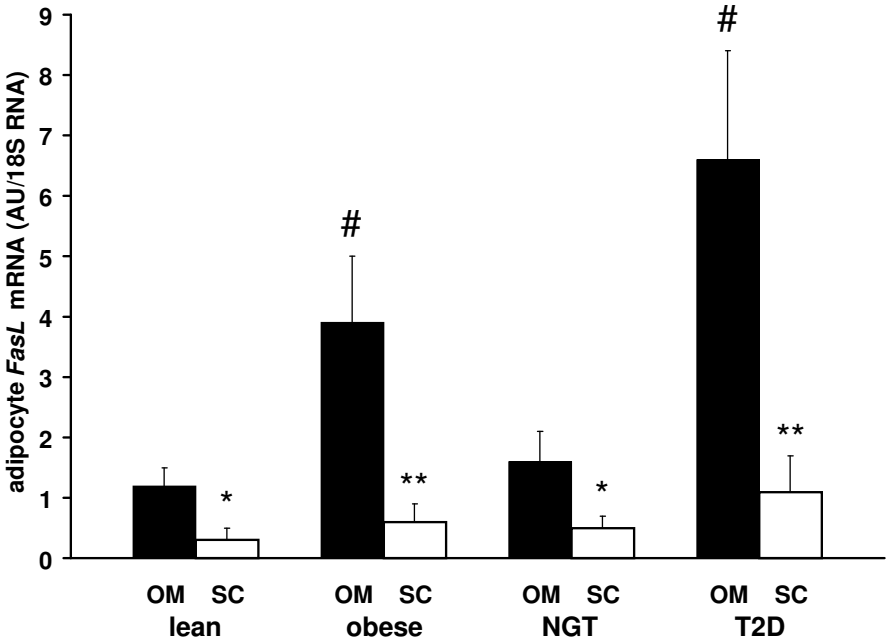
0
**
omental
SC
###
***
Lean
Obese
NGT
T2D
#
*
IS obesity
IR obesity

Figure 3

E


adipocyte FasL mRNA (AU/18S RNA)
9
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OM SC
lean
OM SC
obese
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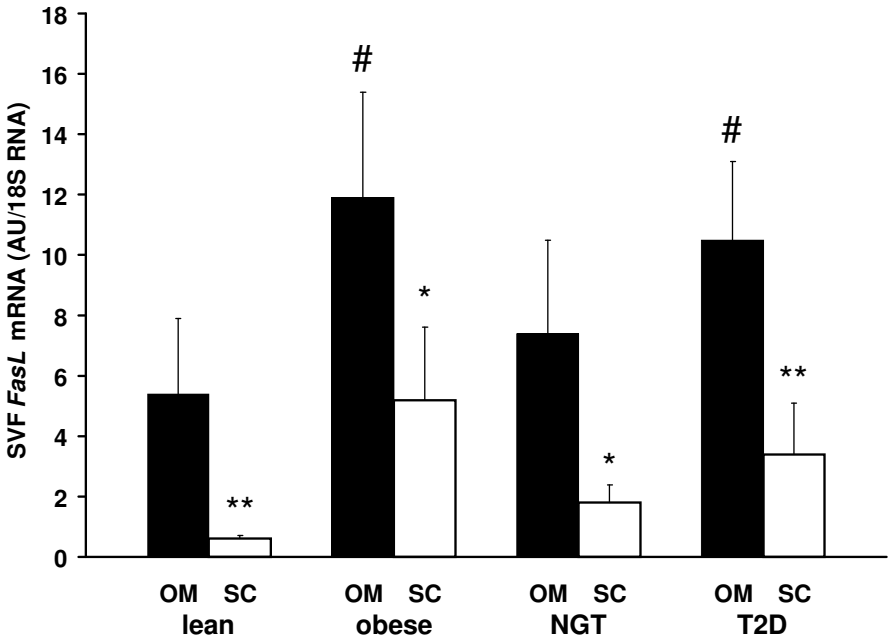
T2D

F


SVF FasL mRNA (AU/18S RNA)
18
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OM SC
lean
OM SC
obese
OM SC
NGT
OM SC
T2D